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AUTHOR: Israeli Ron S; Powell C Thomas; Corr John G; Fair William R; Heston  
Warren D W(a)

AUTHOR ADDRESS: (a)Memorial Sloan-Kettering Cancer Center, 1275 York Ave.,  
Box 334, New York, NY 10021\*\*USA

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McCarley D L; Weiner R S

Department of Medicine, Gainesville VA Medical Center.

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Remarkable advances in the treatment of urologic malignancies have recently been made. Monoclonal antibodies selective for a variety of normal and malignant urologic tissues have been useful in defining normal antigens and tumor-associated antigens and have potential as diagnostic and immunotherapeutic agents. In renal cancer, monoclonal antibodies can define serum markers, radiolabel tumor xenografts, and assist in specific tissue diagnosis. Additionally, there is potential for these antibodies either alone or as conjugates to localize and kill tumors. Monoclonal antibodies to bladder cancer associated antigens are able to demonstrate differential antigen expression on superficial versus invasive tumors, to refine urinary cytologic diagnosis of bladder cancer, and to predict invasive recurrence of superficial cancer. Monoclonal antibodies have localized bladder tumor xenografts and can inhibit tumor growth when conjugated to radioisotopes or toxins. In prostate cancer monoclonal antibodies to prostate antigens are not usually tumor specific. Monoclonal antibodies to prostate antigen (PA) and prostatic acid phosphatase (PAP) are able to localize prostate cancer metastases. Chemotherapy-conjugated anti-PAP monoclonal antibodies have demonstrable inhibition on human prostate cancer xenografted tumor growth. Monoclonal antibodies have defined normal and tumor-associated antigens in urologic cancers and are expected to be useful in immunodiagnosis and cancer therapy in the near future. (63 Refs.)

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# Expression of the Prostate-specific Membrane Antigen<sup>1</sup>

Ron S. Israeli, C. Thomas Powell, John G. Corr, William R. Fair, and Warren D. W. Heston<sup>2</sup>

Urologic Oncology Research Laboratory,<sup>1</sup> Memorial Sloan-Kettering Cancer Center, New York, New York 10021

## ABSTRACT

We have recently cloned a 2.65-kilobase complementary DNA (cDNA) encoding the prostate-specific membrane antigen (PSM) recognized by the 7E11-C5.3 anti-prostate monoclonal antibody. Immunohistochemical analysis of the LNCaP, DU-145, and PC-3 prostate cancer cell lines for PSM expression using the 7E11-C5.3 antibody reveals intense staining in the LNCaP cells with no detectable expression in both the DU-145 and PC-3 cells. Coupled *in vitro* transcription/translation of the 2.65-kilobase full-length PSM cDNA yields an *M*<sub>r</sub> 84,000 protein corresponding to the predicted polypeptide molecular weight of PSM. Posttranslational modification of this protein with pancreatic canine microsomes yields the expected *M*<sub>r</sub> 100,000 PSM antigen. Following transfection of PC-3 cells with the full-length PSM cDNA in a eukaryotic expression vector, we detect expression of the PSM glycoprotein by Western analysis using the 7E11-C5.3 monoclonal antibody. Ribonuclease protection analysis demonstrates that the expression of PSM mRNA is almost entirely prostate specific in human tissues. PSM expression appears to be highest in hormone-deprived states and is hormonally modulated by steroids, with 5- $\alpha$ -dihydrotestosterone down-regulating PSM expression in the human prostate cancer cell line LNCaP by 8–10-fold, testosterone down-regulating PSM by 3–4-fold, and corticosteroids showing no significant effect. Normal and malignant prostatic tissues consistently show high PSM expression, whereas we have noted heterogeneous, and at times absent, expression of PSM in benign prostatic hyperplasia. LNCaP tumors implanted and grown both orthotopically and *s.c.* in nude mice abundantly express PSM, providing an excellent *in vivo* model system to study the regulation and modulation of PSM expression.

## INTRODUCTION

Prostate cancer is among the most significant medical problems in the United States because the disease is now the most common malignancy diagnosed in American males. In 1992, there were over 132,000 new cases of prostate cancer detected with over 36,000 deaths attributable to the disease, representing a 17.3% increase over 4 years (1). Five-year survival rates for patients with prostate cancer range from 88% for those with localized disease to 29% for those with metastatic disease. The rapid increase in the number of cases appears to result in part from an increase in disease awareness as well as the widespread use of clinical markers such as the secreted proteins PSA<sup>4</sup> and PAP (2). We have recently reported the molecular cloning, from the human prostatic adenocarcinoma cell line LNCaP, of a complementary DNA encoding a *M*<sub>r</sub> 100,000 prostate-specific membrane glycoprotein (3), recognized by the anti-prostate monoclonal antibody 7E11-C5.3 (4). The LNCaP cell line was derived from a supraclavicular lymph node of a patient with hormone-refractory prostate cancer (5). Currently, LNCaP cells provide the best *in vitro* model system to study human prostate cancer since they produce all three prostatic

biomarkers, PSA, PAP, and PSM. The cells possess an aneuploid male karyotype with a Y chromosome, express a high affinity androgen receptor, and are hormonally responsive to both testosterone and DHT. Because PSM appears to be a transmembrane glycoprotein, it is considered an attractive target for both antibody-directed imaging and targeting of prostatic tumor deposits (6). In this paper, we demonstrate expression of PSM protein in LNCaP cell membranes and in PC-3 cells transfected with PSM cDNA. In addition, we report the characterization of PSM mRNA expression in human tissues and in response to steroid hormones.

## MATERIALS AND METHODS

**Cells and Reagents.** The LNCaP, DU-145, and PC-3 cell lines were obtained from the American Type Culture Collection. Details regarding the establishment and characteristics of these cell lines have been published previously (5, 7, 8). Unless specified otherwise, LNCaP cells were grown in RPMI 1640 supplemented with L-glutamine, nonessential amino acids, and 5% fetal calf serum (Gibco-BRL, Gaithersburg, MD) in a CO<sub>2</sub> incubator at 37°C. DU-145 and PC-3 cells were grown in minimal essential medium supplemented with 10% fetal calf serum. All cell media were obtained from the MSKCC media preparation facility. Restriction and modifying enzymes were purchased from Gibco-BRL unless otherwise specified. The modified 7E11-C5.3 anti-PSM monoclonal antibody CYT-356 was provided by the Cytogen Corporation, Princeton, NJ. All other chemical reagents were of the highest grade possible and were obtained from Sigma Chemical Company, St. Louis, MO.

**Immunohistochemical Detection of PSM.** We used the avidin-biotin method of detection to analyze prostate cancer cell lines for PSM antigen expression (9). Cell cytopins were made on glass slides using 5 × 10<sup>4</sup> cells/100  $\mu$ l per slide. Slides were washed twice with phosphate-buffered saline and then incubated with the appropriate blocking serum for 20 min. The blocking serum was drained off, and the cells were incubated with diluted 7E11-C5.3 (5  $\mu$ g/ml) monoclonal antibody for 1 h. Samples were then washed with phosphate-buffered saline and sequentially incubated with secondary antibodies for 30 min and with avidin-biotin complexes for 30 min. Diaminobenzidine served as our chromogen and color development followed by hematoxylin counterstaining and mounting. Duplicate cell cytopins were used as controls for each experiment. As a positive control, the anti-cytokeratin monoclonal antibody CAM 5.2 was used following the same procedure described above. Human EJ bladder carcinoma cells served as a negative control.

***In Vitro* Transcription/Translation of PSM Antigen.** Plasmid 55A containing the full-length 2.65-kilobase PSM cDNA in the plasmid pSPORT 1 (Gibco-BRL) was transcribed *in vitro* using the Promega TNT system (Promega Corp., Madison, WI). T7 RNA polymerase was added to the cDNA in a reaction mixture containing rabbit reticulocyte lysate, an amino acid mixture lacking methionine, buffer, and [<sup>35</sup>S]methionine (Amersham, Arlington Heights, IL), and incubated at 30°C for 90 min. Posttranslational modification of the resulting protein was accomplished by the addition of pancreatic canine microsomes into the reaction mixture (Promega Corp.). Protein products were analyzed by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels which were subsequently treated with Amplify autoradiography enhancer (Amersham) according to the manufacturers instructions and dried at 80°C in a vacuum dryer. Gels were autoradiographed overnight at -70°C using Hyperfilm MP (Amersham).

**Transfection of PSM into PC-3 Cells.** The full-length PSM cDNA was subcloned into the pREP7 eukaryotic expression vector (Invitrogen, San Diego, CA). Plasmid DNA was purified from transformed DH5- $\alpha$  bacteria (Gibco-BRL) using Qiagen maxi-prep plasmid isolation columns (Qiagen, Inc., Chatsworth, CA). Purified plasmid DNA (6–10  $\mu$ g) was diluted with 900  $\mu$ l of Optimem media (Gibco-BRL) and mixed with 30  $\mu$ l of Lipofectin

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<sup>2</sup> To whom requests for reprints should be addressed, at Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, Box 334, New York, NY 10021.

<sup>3</sup> Designated George M. O'Brien Urology Research Center.

<sup>4</sup> The abbreviations used are: PSA, prostate-specific antigen; PAP, prostatic acid phosphatase; DHT, 5- $\alpha$ -dihydrotestosterone; PSM, prostate-specific membrane antigen; cDNA, complementary DNA; TS, Tris-HCl; TS-X, Triton-X 100; MSKCC, Memorial Sloan-Kettering Cancer Center.

reagent (Gibco-BRL) which had been previously diluted with 900  $\mu$ l of Optimum media. This mixture was added to T-75 flasks of 40–50% confluent PC-3 cells in Optimum media. After 24–36 h, cells were trypsinized and split into 100-mm dishes containing RPMI 1640 supplemented with 10% fetal calf serum and 1 mg/ml of Hygromycin B (Calbiochem, La Jolla, CA). The dose of Hygromycin B used was determined previously by a time course/dose response cytotoxicity assay. Cells were maintained in this media for 2–3 weeks with changes of media and Hygromycin B every 4–5 days until discrete colonies appeared. Colonies were isolated using 6-mm cloning cylinders and expanded in the same media. As a control, PC-3 cells were also transfected with the pREP7 plasmid alone. RNA was isolated from the transfected cells, and PSM mRNA expression was detected by both RNase protection analysis (described later) and by Northern analysis.

**Western Blot Detection of PSM Expression.** Crude protein lysates were isolated from LNCaP, PC-3, and PSM-transfected PC-3 cells as described previously (10). LNCaP cell membranes were also isolated according to published methods (10). Protein concentrations were quantitated by the Bradford method using the Bio-Rad protein reagent kit (Bio-Rad, Richmond, CA). Following denaturation, 20  $\mu$ g of protein was electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel at 25 mA for 4 h. Gels were electroblotted onto Immobilon P membranes (Millipore, Bedford, MA) overnight at 4°C. Membranes were blocked in 0.15 M NaCl/0.01 M TS plus 5% bovine serum albumin followed by a 1-h incubation with 7E11-C5.3 monoclonal antibody (10  $\mu$ g/ml). Blots were washed 4 times with 0.15 M NaCl/0.01 M TS/0.05% TS-X and incubated for 1 h with rabbit anti-mouse IgG (Accurate Scientific, Westbury, NY) at a concentration of 10  $\mu$ g/ml. Blots were then washed 4 times with TS-X and labeled with  $^{125}$ I-Protein A (Amersham) at a concentration of 1 million cpm/ml. Blots were then washed 4 times with TS-X and dried on Whatman 3MM paper, followed by overnight autoradiography at -70°C using Hyperfilm MP (Amersham).

**Orthotopic and s.c. LNCaP Tumor Growth in Nude Mice.** LNCaP cells were harvested from subconfluent cultures by a 1-min exposure to a solution of 0.25% trypsin and 0.02% EDTA. Cells were resuspended in RPMI 1640 with 5% fetal bovine serum and washed and diluted in either Matrigel (Collaborative Biomedical Products, Bedford, MA) or calcium and magnesium-free Hank's balanced salt solution. Only single cell suspensions with greater than 90% viability by trypan blue exclusion were used for *in vivo* injection. Male athymic Swiss (*nu/nu*) nude mice 4–6 weeks of age were obtained from the MSKCC animal facility. For s.c. tumor cell injection, one million LNCaP cells resuspended in 0.2 ml of Matrigel were injected into the hindlimb of each mouse using a disposable syringe fitted with a 28-gauge needle. For orthotopic injection, mice were first anesthetized with an i.p. injection of pentobarbital and placed in the supine position. The abdomen was cleansed with Betadine, and the prostate was exposed through a midline incision. LNCaP tumor cells (2.5 million) in 0.1 ml were injected directly into either ventral lobe using a 1-ml disposable syringe and a 28-gauge needle. LNCaP cells with and without Matrigel were injected. Abdominal closure was achieved in one layer using Autoclip wound clips (Clay Adams, Parsippany, NJ). Tumors were harvested in 6–8 weeks, confirmed histologically by faculty of the MSKCC Pathology Department, and frozen in liquid nitrogen for subsequent RNA isolation.

**RNA Isolation.** Total cellular RNA was isolated from cells and tissues by standard techniques (11, 12) as well as by using RNazol B (Cinna/Biotech, Houston, TX). RNA concentrations and quality were assessed by UV spectroscopy on a Beckman DU 640 spectrophotometer and by gel analysis. Human tissue total RNA samples were purchased from Clontech Laboratories, Inc., Palo Alto, CA.

**Ribonuclease Protection Assays.** A portion of the PSM cDNA was subcloned into the plasmid vector pSPORT 1 (Gibco-BRL), and the orientation of the cDNA insert relative to the flanking T7 and SP6 RNA polymerase promoters was verified by restriction analysis. Linearization of this plasmid upstream of the PSM insert followed by transcription with SP6 RNA polymerase yields a 400-nucleotide antisense RNA probe, of which 350 nucleotides should be protected from RNase digestion by PSM RNA. This probe was used in Fig. 4. Plasmid IN-20 containing a 1-kilobase partial PSM cDNA in the plasmid pCR II (Invitrogen) was also used for riboprobe synthesis. IN-20 linearized with *Xmn*I (Gibco-BRL) yields a 298 nucleotide antisense RNA probe when transcribed using SP6 RNA polymerase, of which 260 nucleotides should be protected from RNase digestion by PSM mRNA. This probe was used in Figs. 5 and 6. Probes were synthesized using SP6 RNA polymerase

(Gibco-BRL), rNTPs (Gibco-BRL), RNasin (Promega), and [ $^{32}$ P]rCTP (NEN, Wilmington, DE) according to published protocols (13). Probes were purified over NENSORB 20 purification columns (NEN), and approximately 1 million cpm of purified, radiolabeled PSM probe was mixed with 10  $\mu$ g of each RNA and hybridized overnight at 45°C using buffers and reagents from the RPA II kit (Ambion, Austin, TX). Samples were processed as per manufacturer's instructions and analyzed on 5% polyacrylamide/7 M urea denaturing gels using Seq ACRYL reagents (ISS, Natick, MA). Gels were preheated to 55°C and run for approximately 1–2 h at 25 watts. Gels were then fixed for 30 min in 10% methanol-10% acetic acid, dried onto Whatman 3MM paper at 80°C in a Bio-Rad vacuum dryer, and autoradiographed overnight with Hyperfilm MP (Amersham). Quantitation of PSM expression was determined by using a scanning laser densitometer (LKB, Piscataway, NJ).

**Steroid Modulation Experiment.** LNCaP cells (2 million) were plated onto T-75 flasks in RPMI 1640 supplemented with 5% fetal calf serum and grown 24 h until approximately 30–40% confluent. Flasks were then washed several times with phosphate-buffered saline, and RPMI supplemented with 5% charcoal-extracted serum was added. Cells were then grown for another 24 h, at which time dihydrotestosterone, testosterone, estradiol, progesterone, and dexamethasone (Steraloids, Inc., Wilton, NH) were added at a final concentration of 2 nM. Cells were grown for another 24 h, and RNA was then harvested as described previously; PSM expression was analyzed by ribonuclease protection analysis.

## RESULTS

**Immunohistochemical Detection of PSM.** Using the 7E11-C5.3 anti-PSM monoclonal antibody, PSM expression is clearly detectable in the LNCaP prostate cancer cell line but not in the PC-3 and DU-145 cell lines (Fig. 1) in agreement with results published previously (4).

***In Vitro* Transcription/Translation of PSM Antigen.** As shown in Fig. 2, coupled *in vitro* transcription/translation of the 2.65-kilobase full-length PSM cDNA yields a  $M_r$  75,000–80,000 protein species in agreement with the expected protein product from the 750-amino acid PSM open reading frame. We have not investigated the reason for the two bands seen in the first lane. This may represent proteolytic degradation. Following posttranslational modification using pancreatic canine microsomes, the major band observed is a  $M_r$  100,000–110,000 glycosylated protein species consistent with the mature, native PSM antigen.

**Detection of PSM Antigen in LNCaP Cell Membranes and Transfected PC-3 Cells.** PC-3 cells transfected with the full-length PSM cDNA in the pREP7 expression vector were assayed for expression of PSM mRNA by Northern analysis (data not shown). A clone with high PSM mRNA expression was selected for PSM antigen analysis by Western blotting using the 7E11-C5.3 antibody. In Fig. 3, the  $M_r$  100,000 PSM antigen is well expressed in LNCaP membrane fractions and crude cell lysate (Fig. 3, *Lanes 1* and *2*) as well as in PSM-transfected PC-3 cells (Fig. 3, *Lane 4*) but not in native PC-3 cells (Fig. 3, *Lane 3*). This detectable expression in the transfected PC-3 cells (Fig. 3, *Lane 4*) proves that the previously cloned 2.65-kilobase PSM cDNA encodes the antigen recognized by the 7E11-C5.3 anti-prostate monoclonal antibody and that the antigen is being appropriately glycosylated in the PC-3 cells since the antibody recognizes a carbohydrate-containing epitope on PSM.

**PSM mRNA Expression.** Expression of PSM mRNA in normal human tissues was analyzed using ribonuclease protection assays. Tissue expression of PSM appears predominantly within the prostate, with very low levels of expression detectable in human brain and salivary gland (Fig. 4). We have also noted on occasion detectable PSM expression in normal human small intestine tissue; however, this mRNA expression is variable depending upon the specific riboprobe used (data not shown). All samples of normal human prostate and human prostatic adenocarcinoma assayed ( $n = 18$ ) have revealed clearly detectable PSM expression, whereas we have noted generally decreased or absent expression of PSM in tissues exhibiting benign

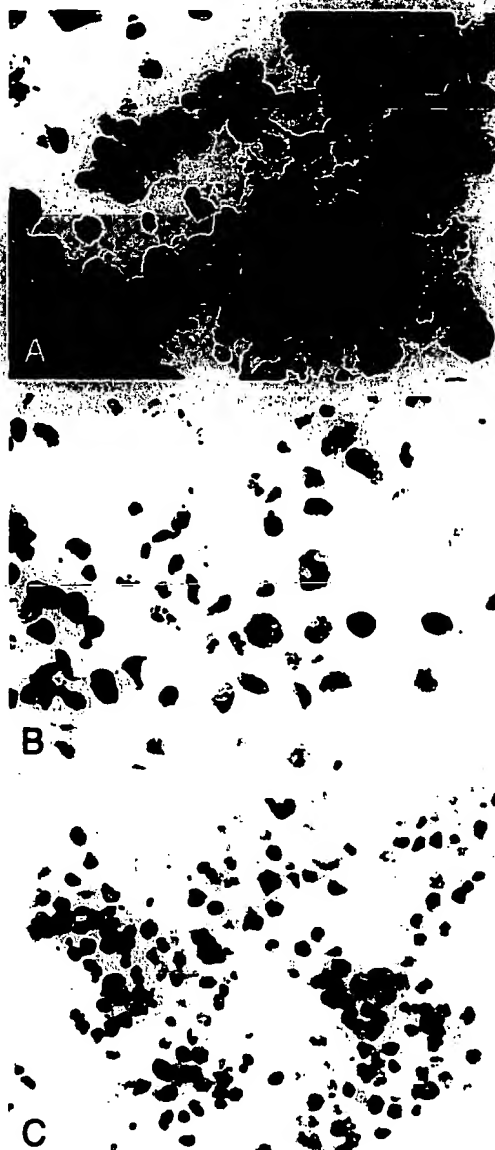


Fig. 1. Immunohistochemical detection of PSM antigen expression in prostate cell lines. Top panel reveals uniformly high level of expression in LNCaP cells; middle and lower panels, DU-145 and PC-3 cells, respectively (both negative).

hyperplasia ( $n = 18$ ; Fig. 5). In human LNCaP tumors grown both orthotopically and s.c. in nude mice, we detected abundant PSM expression with or without the use of Matrigel, which is required for the growth of s.c. implanted LNCaP cells (Fig. 5). Since PSA has been shown to be up-regulated by androgens (14), we investigated the potential androgen responsiveness of PSM which has been noted to increase in expression following hormone deprivation. PSM mRNA expression is distinctly modulated by the presence of steroids in physiological doses (Fig. 6). DHT down-regulated expression by 8–10-fold after 24 h, and testosterone diminished PSM expression by 3–4-fold. Estradiol and progesterone also down-regulated PSM expression in LNCaP cells, perhaps as a result of binding to the mutated androgen receptor known to exist in the LNCaP cell. Overall, PSM expression is highest in the untreated LNCaP cells grown in steroid-depleted media, a situation that we propose simulates the hormone-deprived (castrate) state *in vivo*. This experiment was repeated at steroid dosages ranging from 2–200 nM and at time points from 6 h to

7 days with similar results; maximal down-regulation of PSM mRNA was seen with DHT at 24 h at doses of 2–20 nM (data not shown). A separate RNase protection assay was performed using a human acidic ribosomal phosphoprotein PO probe (15) in the same reaction as the PSM probe. It was noted that the expression of the PO mRNA was not affected by steroid treatment, whereas the changes in PSM expression were identical to those in Fig. 6 (data not shown).

## DISCUSSION

In order to better understand the biology of the human prostate in both normal and neoplastic states, we need to enhance our knowledge

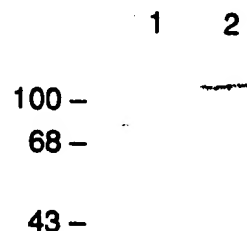


Fig. 2. Autoradiogram of protein gel revealing products of PSM coupled *in vitro* transcription/translation. Nonglycosylated PSM polypeptide is seen at approximately  $M_r$  75,000–80,000 (Lane 1), and PSM glycoprotein synthesized following the addition of microsomes is seen at  $M_r$  100,000–110,000 (Lane 2).



Fig. 3. Western blot analysis detecting PSM expression in transfected non-PSM expressing PC-3 cells.  $M_r$  100,000 PSM glycoprotein species is clearly seen in LNCaP membranes (Lane 1), LNCaP crude lysate (Lane 2), and PSM-transfected PC-3 cells (Lane 4) but is undetectable in native PC-3 cells (Lane 3).

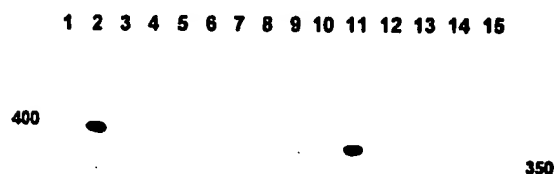


Fig. 4. Autoradiogram of ribonuclease protection gel assaying for PSM mRNA expression in normal human tissues. Lane 1, radiolabeled 1-kilobase DNA ladder (Gibco-BRL). Lane 2, undigested probe is 400 nucleotides and expected protected PSM band is 350 nucleotides. Lane 3, (RNA control). A strong signal is seen in human prostate (Lane 4), with very faint but detectable signals seen in human brain (Lane 5) and human salivary gland (Lane 12). Other samples include Lane 6, kidney; Lane 7, liver; Lane 8, lung; Lane 9, mammary gland; Lane 10, pancreas; Lane 11, placenta; Lane 12, salivary gland; Lane 13, skeletal muscle; Lane 14, spleen; and Lane 15, testis.

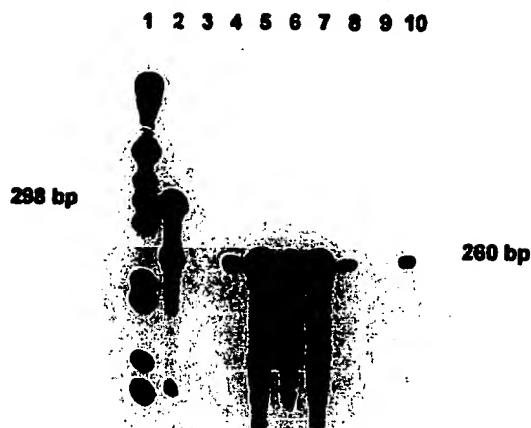


Fig. 5. Autoradiogram of ribonuclease protection assay for PSM mRNA expression in LNCaP tumors grown in nude mice and in human prostatic tissues. Lane 1,  $^{32}$ P-labeled 1-kilobase DNA ladder. Lane 2, 298-nucleotide undigested probe. Lane 3, tRNA control. A 260–270 nucleotide band in the undigested probe lane (Lane 2) is seen and probably represents a partial RNA transcript. This RNA is completely degraded by RNase in the absence of PSM mRNA as seen in the tRNA control (Lane 3). PSM mRNA expression is clearly detectable in LNCaP cells (Lane 4), orthotopically grown LNCaP tumors in nude mice with and without Matrigel (Lanes 5 and 6), and s.c. implanted and grown LNCaP tumors in nude mice (Lane 7). PSM mRNA expression is also seen in normal human prostate (Lane 8) and in a moderately differentiated human prostatic adenocarcinoma (Lane 10). Very faint expression is seen in a sample of human prostate tissue with benign hyperplasia (Lane 9).



Fig. 6. Ribonuclease protection assay for PSM expression in LNCaP cells treated with physiological doses of various steroids for 24 h. Lane 1,  $^{32}$ P-labeled DNA ladder. Lane 2, 298-nucleotide undigested probe. Lane 3, tRNA control. PSM mRNA expression is highest in untreated LNCaP cells in charcoal-stripped media (Lane 4). We see significantly diminished PSM expression in LNCaP cells treated with DHT (Lane 5), testosterone (Lane 6), estradiol (Lane 7), and progesterone (Lane 8), with little response to dexamethasone (Lane 9).

by studying the various proteins and other features that are unique to this important gland. Previous research has provided two valuable prostatic biomarkers, PAP and PSA, both of which have had a significant impact on the diagnosis, treatment, and management of prostate malignancies. Our present work describing the preliminary characterization of the PSM reveals it to be a gene with many interesting features. PSM is almost entirely prostate specific, as are PAP and PSA, and as such may enable further delineation of the unique functions and behavior of the prostate. The predicted sequence of the PSM protein (3) and its presence in the LNCaP cell membrane as determined by Western blotting and immunohistochemistry indicate that it is an integral membrane protein. Thus, PSM provides an attractive cell surface epitope for antibody-directed diagnostic imaging and cytotoxic targeting modalities.<sup>5</sup> The ability to synthesize the

PSM antigen *in vitro* and to produce tumor xenografts maintaining high levels of PSM expression provides us with a convenient and attractive model system to further study and characterize the regulation and modulation of PSM expression. Also, the high level of PSM expression in the LNCaP cells provides an excellent *in vitro* model system. Since PSM expression is hormonally responsive to steroids and may be highly expressed in hormone-refractory disease (16), it is imperative to elucidate the potential role of PSM in the evolution of androgen-independent prostate cancer. The detection of PSM mRNA expression in minute quantities in brain, salivary gland, and small intestine warrants further investigation, although these tissues were negative for expression of PSM antigen by immunohistochemistry using the 7E11-C5.3 antibody (17). In all of these tissues, particularly small intestine, we detected mRNA expression using a probe corresponding to a region of the PSM cDNA near the 3' end, whereas we were unable to detect expression when using a 5' end PSM probe. These results may indicate that the PSM mRNA transcript undergoes alternative splicing in different tissues. Previous protein studies have suggested that the 7E11-C5.3 antibody may actually detect two other slightly larger protein species in addition to the  $M_r$  100,000 PSM antigen (18). These other protein species can be seen in the LNCaP lysate and membrane samples in Fig. 3. Possible origins of these proteins include alternatively spliced PSM mRNA, other genes distinct from but closely related to PSM, or different posttranslational modifications of the PSM protein. We are currently investigating these possibilities.

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